Antibodies to Mycobacterial Peptidoglycolipid and to Crude Protein Antigens in Sera from Different Categories of Human Subjects

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Sera from patients with disease caused by the Mycobacterium avium complex (M. avium and M. intracellulare), M. kansasi, or M. tuberculosis and from subjects who did not have a mycobacterial disease were tested by enzyme-linked immunosorbent assay against peptidoglycolipid antigens representing each of the 15 most common serovars of the M. avium complex and against crude protein antigen extracts of M. avium and M. tuberculosis. The highly specific peptidoglycolipid antigens yielded positive reactions in 83% of M. avium complex patients, 57% of active-tuberculosis patients, and 14% of subjects without mycobacterial disease. Reactions to more than 1 of the 15 peptidoglycolipid antigens were found only in patients with infections caused by mycobacteria, suggesting that a mycobacterial pulmonary lesion is readily colonized by mycobacteria other than the one that initiated the lesion. The two crude mycobacterial protein antigens were highly cross-reactive, with little if any capacity to discriminate between infections caused by any of the mycobacteria studied. Moreover, they did not appear to be more sensitive than the peptidoglycolipids. The data suggest that it is unlikely that a practical and reliable serological test can be developed that will distinguish between transient subclinical infection and significant disease caused by common environmental mycobacteria, such as members of the M. avium complex. Success in developing such a test for nonenvironmental mycobacteria, such as M. tuberculosis, appears more likely.

The search for a satisfactory serological test for tuberculosis and other mycobacterial diseases presents a difficult challenge in balancing sensitivity against specificity (14). Proteins of most species of mycobacteria, both environmental opportunists and strict pathogens, share many epitopes (5, 6, 8). For example, antibodies to the T class of catalase from one mycobacterial species cross-react with different species that produces that enzyme, and the same holds true for M catalase (19). However, cross absorption of sera yields antibodies that react only with the catalase of members of the homologous species (20, 21), thus proving the presence of both epitopes that are species specific and epitopes that are common to the other members of the genus, all on the same molecule. Hewitt and colleagues (11) used monoclonal antibodies to demonstrate epitopes that were unique to proteins of Mycobacterium tuberculosis; some were so specific as to be absent in some strains of that species. Other epitopes were common to two or more species but not to all strains of a reactive species. The application of a monoclonal antibody in competitive assays to detect antibody in sera of human subjects yielded positive results in only 74% of patients with culturally proven active tuberculosis (13).

In contrast to the serologic cross-reactivity of mycobacterial proteins, a very high degree of specificity exists in the unbound surface peptidoglycolipids and other glycolipids that are responsible for the infrasubspecific serovar agglutination classification of a number of mycobacterial species (1, 2, 15). For example, strains of M. avium, M. intracellulare, and M. scrofulaceum all produce a surface antigen with a common peptidoglycolipid core, and the specificity of the 30 or more serovars within the serocomplex is determined by the composition of the oligosaccharide linked at a defined point on that core (1, 2). Dawson and colleagues (9) demonstrated the presence of serovar-specific agglutinating antibody to members of the M. avium complex in the sera of five patients from whom the organisms were isolated. Wayne and colleagues (23) demonstrated specific antibody against purified serovar-specific peptidoglycolipid antigen by enzyme-linked immunosorbent assay (ELISA) in sera from a small series of patients with pulmonary infection caused by M. avium complex but not in sera of acquired immunodeficiency syndrome patients with proven disseminated M. avium complex infections. M. leprae produces a unique phenolic glycolipid surface antigen that has been exploited in the development of a sensitive and specific serological test for leprosy (7).

Many evaluation studies of serological tests for tuberculosis used as controls sera from subjects who either were healthy or had some nonmycobacterial disease. It was the purpose of the present study to compare results of serological testing of sera from populations with different mycobacterial diseases with results of sera from patients with nonmycobacterial pulmonary and nonpulmonary disease, as well as healthy controls, using both relatively nonspecific crude protein antigens and purified highly specific peptidoglycolipid antigens. Although neither of these two types of antigens proved to be of sufficient diagnostic value to be adopted for routine testing of clinical materials, the results of the study provide some insights into the natural history of mycobacterial infection.

MATERIALS AND METHODS

Serologic reference materials. Rabbit antisera to each of the agglutinating serovars of M. avium and M. intracellulare were provided by Anna Tsang, National Jewish Hospital and Research Center, Denver, Colo., with support from the United States—Japan Cooperative Medical Sciences Program. The M. avium complex comprises the two species M. avium and M. intracellulare, and each of these species
comprises 10 or more agglutinating serovars (21). Selected strains of each of the desired serovars to be used for preparation of polar peptidoglycolipid antigens were provided by Robert C. Good, Centers for Disease Control, Atlanta, Ga.

**Human sera.** Sera from patients with different diseases (see footnote to Table 1) were obtained at this medical center. Some additional sera from patients with *M. avium* complex infections were also provided through the courtesy of Bonnie Lutz, Orange County Health Department, Santa Ana, Calif., and David Salkin, La Vina Sanatorium, Altadena, Calif. Sera from healthy blood donors and information on ages of the donors were provided by Harold Kaplan and Areli Shapiro, Orange County Chapter of the American Red Cross. The diagnosis of disease caused by mycobacteria other than *M. tuberculosis* was based on clinical signs and symptoms compatible with those infections, with more than one isolation of the mycobacterial agent and no isolations of *M. tuberculosis* from the patients’ specimens.

**Preparation of polar peptidoglycolipid antigens.** Strains of *M. avium* and *M. intracellulare* representing each of the 15 serovars to be tested were grown for 2 to 3 weeks on Dubos oleic albumin agar. The bacilli were harvested and washed twice in distilled water, and the volume of packed cells was recorded. The extraction procedure was based on methods described by Brennan and colleagues (2, 3). Volumes of reagents represented the amounts used per milliliter of packed bacilli. The cells were extracted overnight at room temperature in 12 ml of a 1:1 mixture of chloroform and methanol and centrifuged. The supernatant solution was removed, and the cells were re-extracted in 4 ml of 1:1 chloroform-methanol. The two supernatant extracts were combined, and the pooled solution was resolved into two phases with 2.0 ml of phosphate-buffered saline, pH 7.5. The aqueous-methanol phase was discarded, and the chloroform phase was removed from any solid residue that formed and taken to dryness at 50°C in a stream of nitrogen. The residual solid was taken up in 5 ml of dry chloroform and centrifuged to remove insoluble residue. The chloroform was evaporated to dryness, and the residue was taken up in 5 ml of dry methanol and centrifuged. Chloroform (10 ml) was added to the methanol extract, and the solution was warmed to 37°C.

The antigens were deacylated by adding 15 ml of prewarmed 0.2 M NaOH in methanol and incubating at 37°C for 20 min, and the hydrolysis was stopped with 0.375 ml of glacial acetic acid. Normal saline (12 ml) was added to break the solution into two phases. The chloroform phase was withdrawn, washed with 2.5 ml of normal saline, and taken to dryness as before. The dried solute was extracted with hexane at 50°C for 30 min to remove nonpolar peptidoglycolipid, and the hexane-insoluble residue, which comprised the desired polar peptidoglycolipid antigen, was dissolved in 5 ml of absolute ethanol. Samples were assayed for sugar content by the method of Dubois et al. (10) with rhomannose as the standard. Concentrations of antigen were expressed as micrograms of rhomannose equivalents per milliliter.

**Preparation of crude protein antigens.** The bacilli were grown in Dubos broth base (Difco Laboratories, Detroit, Mich.) containing 1% (wt/vol) glycerol and enriched with Dubos medium albumin (Difco) for *M. tuberculosis H37Rv* or with Dubos oleic albumin complex (Difco) for *M. avium* SBJ-2 (serovar 8). The methods of growth, harvest, sonication, and separation of crude bacillary protein from the bulk of the lipids by absorption onto and elution from DEAE-cellulose (Sigma Chemical Co., St. Louis, Mo.) were described previously (22). Protein concentrations of the extracts were determined with the Coomassie brilliant blue protein-binding reagent (Bio-Rad Laboratories, Richmond, Calif.).

**ELISA technique.** Dilutions of peptidoglycolipid antigens were titrated against specific rabbit antisera to the different serovars; a load of 0.025 µg of antigen per well was found to represent an excess, such that the optical absorbances after addition of final test substrate were a direct function of the logarithm of the serum dilutions, within the upper limits of response of the enzyme immunoassay reader. For testing human sera, the peptidoglycolipid antigens were dissolved in ethanol to a final concentration of 1.25 µg of rhomannose equivalents per ml. Twenty-microtiter samples of diluted antigens, corresponding to 0.025 µg of rhomannose equivalents, were drawn by pipette to wells of polystyrene Nunc II immunoplates (Vanguard International, Neptune, N.J.) and dried at 37°C. The wells were then filled with 0.05 M bicarbonate buffer (pH 9.4) and held at room temperature overnight. The next morning the buffer was aspirated, and the wells were blocked for 2 h at 37°C with bovine serum albumin (10 mg/ml in phosphate-buffered saline), preserved with 1:10,000 thimerosal (albumin-phosphate-buffered saline). After being washed one more time with albumin-phosphate-buffered saline, the plates could be used immediately or after storage at 5°C for 1 week or more.

For tests with crude protein antigens, the wells were primed by the addition of 20 µl of a solution containing 100 µg of the desired protein per ml and 100 µl of 0.05 M bicarbonate buffer (pH 9.4) and held at room temperature overnight. The wells were then aspirated and blocked by the same procedure as used for the peptidoglycolipid-primer plates described above.

For the surveys of human sera against either protein or peptidoglycolipid antigens, 100 µl of a 1:100 dilution of human serum was dispensed to wells coated with each of the selected antigens and to a blocked well without antigen and incubated at room temperature for 2 h. The serum was then aspirated, and the wells were washed three times with a solution of 0.05% Tween 20 in normal saline (Tween-saline) and once in plain saline.

The second antibody, alkaline phosphatase-conjugated goat anti-human immunoglobulin G (or anti-rabbit immunoglobulin G, for standardization of reagents against specific rabbit sera) (Miles Scientific, Naperville, Ill.) was diluted in 0.05 M Tris buffer (pH 8.0) containing (per liter) MgCl₂, 6H₂O, 200 mg; bovine albumin V, 1.0 g; and sodium azide, 200 mg; the concentration of conjugate used gave a full-scale reading at 405 nm on an enzyme immunoassay reader (model EL307; Bio-Tek Instruments, Inc., Burlington, Vt.) when tested against wells that had been primed with a saturating amount of human immunoglobulin G, followed by substrate. Each test well received 250 µl of diluted second antibody and was incubated at room temperature for 2 h. After aspiration of the second antibody, the wells were washed four times with Tween-saline and three times with plain saline.

Each well received 250 µl of substrate solution (0.4 mg of 3-μnitrophenyl phosphate per ml in 0.75 M 2-amino-2-methyl-1-propanol buffer [pH 10.3]), and the plates were incubated for 1 h at room temperature. The reaction was stopped with the addition of 50 µl of 0.3 M NaOH, and the A₄₀₅ was recorded.

**Statistical tests.** The statistical significance of differences between serum raw score distributions from different categories of human subjects was determined with the nonparametric rank order Mann-Whitney U test (17).
RESULTS

The human sera were classified according to the categories of subjects from whom they were obtained (see footnote to Table 1). Sera from a total of 107 subjects were tested by ELISA against peptidoglycolipid antigens extracted from strains representing the 15 most frequently encountered serovars of the *M. avium* complex, i.e., 1, 2, 4, 6, 7, 8, 9, 10, 12, 13, 14, 16, 18, 19, and 25 (15). Several sera were tested repeatedly, both to assess reproducibility of response and to serve as positive and negative controls for survey plates. Means and standard deviations of replicates of raw data are presented for selected serum specimens in Fig. 1 and demonstrate satisfactory reproducibility of the assay system. The extent of nonspecific background binding of immunoglobulin varied markedly from serum to serum, but specific reaction peaks were evident. For example, the mean $A_{405}$ values of eight replications of serum 20 (Fig. 1A) against all 15 antigens and in the antigen-free background well were all below 0.10 and within one standard deviation of one another. In contrast, the mean $A_{405}$ values of eight replications of serum 55, from a patient with *M. avium* complex infection (Fig. 1C), were high and variable for the different antigen wells. This did not appear to represent cross-reaction to different antigens but rather some nonspecific adherence of the immunoglobulin to the plastic or the blocking albumin, since the mean $A_{405}$ for the antigen-free well was higher than that for those containing antigens of 11 of the serovars. A similarly high antigen-free background absorbance was seen with serum 74, from another *M. avium* complex patient (Fig. 1D); whereas serum 61, from a third *M. avium* complex patient (Fig. 1B), yielded a mean antigen-free well $A_{405}$ almost identical to those in 14 of the antigen wells. All three of the sera from *M. avium* complex patients shown in Fig. 1 gave peak reactions to serovar 8, but serum 55 (Fig. 1C) also showed peaks to serovar 7, whereas serum 74 (Fig. 1D) also peaked with serovars 4 and 10. These cases appear to represent multiple specificities rather than cross-reactions, since each serum gave a different pattern, even though all three reacted to serovar 8.

As noted above, there was considerable variation in nonspecific adherence of the immunoglobulins from serum to serum, and the antigen-free well did not always yield the lowest score to serve as a background reading for some of the specimens. For this reason, the following formula was adopted for recording the ELISA score ($\Delta A$) for each peptidoglycolipid antigen: $\Delta A = A_{405} - M$, where $A_{405}$ is the raw $A_{405}$ in a given antigen well and $M$ is the mean $A_{405}$ for all 15 of the antigen-containing wells in a given serum row. The highest $\Delta A$ score seen with each serum against the 15 peptidoglycolipid antigens was recorded as $\Delta A_{\text{max}}$, and the distribution of $\Delta A_{\text{max}}$ scores was plotted for each of the human subject categories (Fig. 2).

The highest scores against one or more of the 15 serovar-specific peptidoglycolipid antigens of the *M. avium* complex were seen with sera from patients with pulmonary disease caused by members of the *M. avium* complex (MAC); the difference in score distribution was statistically significant compared with that of all other categories of human subjects ($P < 0.05$ compared with sera from the active-tuberculosis patients [TBA]; $P < 0.025$ compared with all other categories). The second highest score distribution was seen with sera from patients with TBA, and here also the difference in score distribution was significant when compared with all other categories ($P < 0.025$ compared with sera from cardiac disease patients [CAR]; $P < 0.01$ compared with all other categories). The differences in $\Delta A_{\text{max}}$ distributions among the remaining four subject categories, inactive-tuberculosis patients (TBX), *M. kansasi* patients (MKA), chronic obstructive pulmonary disease patients (COP), and healthy blood donors (NOR), were not statistically significant ($P \geq$
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pid antigens, according with each function of distribution and that of TBA the ELISA is frequencies 1. Table were positive approximately M. kansasii according to disease mycobacterial 15 the and 25) antibody antigen species M. gens among disease. occurrence was groups (+ ages years, respectively. £A4max of significantly members <r COP

FIG. 2. Distribution of highest ELISA score (ΔA max) observed with each serum against any of the serovar-specific peptidoglycolipid antigens, according to category of human subjects. See footnote to Table 1 for category codes.

0.05). However, inspection of Fig. 2 suggests that the score distribution in the MKA group is higher than those from any of the subjects who did not have known mycobacterial disease and that the lack of statistical significance may be a function of the small number of subjects in this category.

If the reaction of a given serum in the peptidoglycolipid ELISA is interpreted as positive for those antigens for which the ΔA was ≥0.20, then 85% of the MAC sera, 57% of the TBA sera, 50% of the MKA sera, and 30% of the TBX sera were positive against one or more of the 15 peptidoglycolipid antigens tested, and fewer than 20% of any of the other sera exceeded that threshold (COP, 18%; CAR, 14%; NOR, 8%). The distribution of all positive reactions (ΔA, ≥0.20) according to subject category and serovar is presented in Table 1 to illustrate both the relative frequencies of responses to each of the individual antigens and the relative frequencies of responses to more than one antigen. Approximately half of the positive sera from patients with active disease caused by M. avium complex, M. tuberculosis, or M. kansasii exhibited ΔA scores of ≥0.20 with peptidoglycolipid antigens of two or more different serovars, whereas none of the sera from subjects who did not have active mycobacterial disease gave multiple positive reactions. Of the 15 peptidoglycolipid antigens tested, 7 (serovars 1, 2, 4, 6, 8, 9, and 10) are now considered to be included in the species M. avium and 8 (serovars 7, 12, 13, 14, 16, 18, 19, and 25) belong to M. intracellulare (21). Of a total of 52 positive reactions, 26 were to M. avium serovars and 28 were to serovars of M. intracellulare. The M. avium serovar 8 antigen accounted for 19 (37%) of all positive reactions, including all subject categories. The second most frequent response was to M. intracellulare serovar 14 (nine cases), but antibody to this serovar was found only in patients with mycobacterial disease.

The data were also reviewed to determine whether the occurrence of positive reactions to peptidoglycolipid antigens among subjects with no known mycobacterial infection was a function of the age of the subjects. The mean ages (± standard deviation) of the COP, CAR, and NOR groups were 66.1 (±10.7), 63.9 (±11.7), and 35.9 (±14.1) years, respectively. It was noted above that the distributions of ΔA max scores among these three groups were not significantly different from one another. Approximately half of the members of the COP and CAR groups and none of the NOR group were over 65 years of age. When the comparison of CAR and COP subjects was restricted to subjects who were over 65 years of age, the difference in distribution of ΔA max scores between these two control groups was not significant (P > 0.05); however, the scores of COP subjects who were under 65 years old were significantly lower than those of COP subjects who were older (P < 0.05), but a comparable age-related distinction could not be demonstrated in the CAR group.

Crude protein antigens from M. tuberculosis and M. avium in ELISA showed little if any discrimination between sera from patients with active disease caused by M. tuberculosis and M. avium complex, as reflected by the lines of regression comparing these antigens against sera from each of these two human subject categories (Fig. 3).

The distribution of ELISA scores was plotted for these two antigens for all human subject categories (Fig. 4). The distribution of ELISA scores of sera from patients with TBA was not significantly different (P > 0.05) from that from patients infected with MAC, regardless of whether crude protein from M. tuberculosis or M. avium was used as the antigen. The distributions of ELISA scores from patients with active disease caused by M. tuberculosis or the M. avium complex were significantly higher than those from subjects with no known mycobacterial disease (P values

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* CAR, Cardiac disease; no known mycobacterial infection; COP, chronic obstructive pulmonary disease; no known mycobacterial infection; MAC, active pulmonary disease associated with M. avium complex; M. tuberculosis never isolated; MKA, active pulmonary disease associated with M. kansasii complex; M. tuberculosis never isolated; NOR, healthy control subjects; TBA, pulmonary tuberculosis; M. tuberculosis isolated within preceding 2 years; TBX, past history of pulmonary tuberculosis; no cavity present and M. tuberculosis not isolated within preceding 2 years.
ranging from <0.0001 to <0.05 in all cases), regardless of which of the two crude protein antigens was used. Furthermore, sera from subjects with no known mycobacterial disease but with ΔA_{max} scores of ≥0.20 against a peptidoglycolipid surface antigen did not exhibit significantly higher scores against the crude protein antigens than those of sera from subjects with no known mycobacterial disease that gave low ΔA_{max} scores against all peptidoglycolipids tested.

**DISCUSSION**

The peptidoglycolipid surface antigens from the *M. avium* complex were selected for this study because of their striking specificity, whereas the crude protein extracts of *M. avium* and *M. tuberculosis* were used to present the maximum number of different mycobacterial epitopes to the antibodies with the intent of favoring sensitivity. As seen in Fig. 2, sera from the patients with pulmonary disease caused by members of the *M. avium* complex gave the highest responses to one or more of the peptidoglycolipid antigens, with 83% exhibiting ΔA_{max} scores exceeding 0.20. Some of the sera that did not react may have been from patients infected with serovars that were not included among the 15 selected for this study; these 15 serovars, in the aggregate, accounted for 87.6% of the strains of *M. avium* and *M. intracellularare* in the frequency table published by McClatchy et al. (15). Furthermore, 56% of the patients with pulmonary disease caused by mycobacteria other than members of the *M. avium* complex, i.e., the combined TBA and MKA groups, yielded ΔA_{max} scores above 0.20, whereas only 14% of the subjects who had no known mycobacterial disease, i.e., the combined COP, CAR, and NOR groups, gave scores above this threshold with peptidoglycolipid antigens. It is of special interest that over half of the patients infected with *M. tuberculosis* or *M. kansasii*, neither of which produces the peptidoglycolipid antigens of the *M. avium* complex, displayed significant levels of antibody to one or more of these antigens. Tsukamura and colleagues (18) reported the isolation of members of the *M. avium* complex in mixed culture from more than 10% of sputum specimens that yielded *M. tuberculosis*. They speculated that coinfection may occur at the time of primary infection, but it appears equally likely that these ubiquitous organisms colonize pulmonary cavities that result from initial infection with *M. tuberculosis*.

The appearance of positive reactions to peptidoglycolipid
antigens representing two or more different serovars in the sera of a number of patients with known pulmonary mycobacterial disease but not of any of the subjects without mycobacterial disease, or of any patients whose tuberculosis had been inactive for 2 or more years (Table 1). The occurrence of a positive reaction to a single peptidoglycolipid antigen in sera from 14% of human subjects with no history of mycobacterial disease is consistent with transient subclinical infection by these organisms from the environment. Palmer and Edwards (16) reported sensitization to a skin test antigen prepared from a member of the M. avium complex in proportions ranging from <20 to >70% of U.S. Navy recruits, depending on their geographic region of origin. The low levels of antibody to any of the mycobacterial antigens in our ELISAs of most of the sera from subjects whose tuberculosis had been inactive for 2 years or more suggest that the serologic B-cell response is of shorter duration than the T-cell response associated with the skin test, in agreement with observations of Huygen and colleagues (12), who used purified protein derivative and a 32-kilodalton protein.

When crude protein extracts were used as antigens, there was no practical difference between proportional responses to proteins from M. tuberculosis and M. avium, regardless of which organism was responsible for the subjects' disease (Fig. 4). Although these extracts contained innumerable epitopes associated with a multiplicity of proteins, the distribution of positive responses (i.e., ELISA scores above the selected threshold A_{405} of 0.20) for the subjects who did not have mycobacterial diseases (CAR, COP, and NOR) was comparable to that seen when only the 15 highly defined small epitopes on peptidoglycolipid antigens were offered. The tuberculosis patients showed a small increase in numbers of positive results when crude protein was used instead of peptidoglycolipid antigen, presumably accounting for subjects who were infected with M. tuberculosis but not superinfected with a member of the M. avium complex.

The data presented here allow some inferences to be drawn about the prospect of developing useful immunologic tests for diagnosis of mycobacterial diseases. The positive serologic responses to peptidoglycolipid antigens representing single serovars of the M. avium complex by many human subjects who had no demonstrable disease caused by a member of that complex do not suggest a lack of specificity in terms of ability to recognize very specific, well-defined mycobacterial epitopes. Rather, they are evidence of a lack of serologic discrimination between transient infection and significant disease caused by opportunistic environmental mycobacteria, such as those of the M. avium complex. This conclusion is supported by the similarity in distributions of ELISA scores among subjects without known mycobacterial disease whether tested against the very specific M. avium complex peptidoglycolipids or against very crude protein extracts of M. avium. These results suggest that it may not be possible to develop a reliable serological test that is diagnostic of disease caused by common environmental mycobacteria. In contrast, the phenolic glycolipid surface antigen of M. leprae has been the basis of a very useful test for the diagnosis of leprosy (4, 7); that organism is not likely to be found free in the environment. M. tuberculosis is also not a free-living organism in the environment, so it is likely that a suitable antigen and test strategy that will be both sensitive and specific enough to permit the diagnosis of tuberculosis can be developed. The tendency of the antibody response to fade after the disease has become inactive suggests that such a test could reflect the state of activity of the disease.

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LITERATURE CITED

avium-Mycobacterium intracellulare complex together in the sputum of patients with pulmonary tuberculosis. Tubercle 62: 43–46.